

CAZyme content of *Pochonia chlamydosporia* reflects that chitin and chitosan modification are involved in nematode parasitism

Authors:

Almudena Aranda-Martinez^{1*}, Nicolas Lenfant², Nuria Escudero¹, Ernesto A. Zavala-Gonzalez¹, Bernard Henrissat^{2,3,4} and Luis V. Lopez-Llorca¹.

Affiliations:

¹Laboratory of Plant Pathology, Department of Marine Sciences and Applied Biology, Multidisciplinary Institute for Environmental Studies Ramón Margalef, University of Alicante, Alicante, Spain.

²Architecture et Fonction des Macromolécules Biologiques, CNRS, Aix-Marseille Université, Marseille, France.

³INRA, USC 1408 AFMB, Marseille, France.

⁴Department of Biological Sciences, King Abdulaziz University, Jeddah, Saudi Arabia.

*Corresponding author:

Almudena Aranda-Martinez

Laboratory of Plant Pathology, Department of Marine Sciences and Applied Biology, Multidisciplinary Institute for Environmental Studies Ramón Margalef, University of Alicante, P.O. Box 99, E-03080, Alicante, Spain.

Tel. (+34) 965903400 ext 2223

Fax (+34) 965909897

almudena.aranda@ua.es

Running title:

CAZome of *Pochonia chlamydosporia*

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Originality-Significance Statement

Plant parasitic nematodes (PPN) endanger food security (FS). Their management with organophosphates and fumigants causes damage to the environment and human health.

The nematophagous fungus *Pochonia chlamydosporia* (Pc) is the main cause of natural soil suppression to PPN. Induction of suppressivity is a key factor for the success of microbial inoculants such as Pc. Pc is also an endophyte which enhances growth and yield of FS crops such as tomato. Our analyses of the CAZymes encoded by *Pochonia chlamydosporia* genome explain the multitrophic lifestyle of Pc. Just like that of plant saprotrophs and pathogens and unlike fungi very close phylogenetically such as the entomopathogen *Metarhizium anisopliae*, Pc genome encodes enzymes to degrade the key components of the plant cell wall. However the most striking feature of Pc CAZome is the enrichment of chitin but specially chitosan degrading enzymes (GH75). This is validated by the large capacity of Pc to withstand and degrade this polymer which is inhibitory or lethal for other fungi. We have detected chitosan synthesis in PPN eggs infected by Pc. In accordance with this, CE4 (chitin deacetylase) but also GH75 (chitosanase) encoding genes are induced during the infection of nematode eggs by Pc. We propose that chitosan synthesis and degradation is a new mechanism involved in the parasitism of nematode eggs and therefore important for the natural suppression of soils to PPN. CE4 and GH75 may act together with other hydrolases (chitinases and serine proteases) to degrade the egg shell, the most resistant structure of PPN to environmental stress.

Summary

Pochonia chlamydosporia is a soil fungus with a multitrophic lifestyle combining endophytic and saprophytic behaviors, in addition to a nematophagous activity directed

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against eggs of root-knot and other plant parasitic nematodes. The carbohydrate-active enzymes encoded by the genome of *P. chlamydosporia* suggest that the endophytic and saprophytic lifestyles make use of a plant cell wall polysaccharide degradation machinery that can target cellulose, xylan and, to a lesser extent, pectin. This enzymatic machinery is completed by a chitin breakdown system that involves not only chitinases, but also chitin deacetylases and a large number of chitosanases. *P. chlamydosporia* can degrade and grow on chitin and is particularly efficient on chitosan. The relevance of chitosan breakdown during nematode egg infection is supported by the immunolocalization of chitosan in *Meloidogyne javanica* eggs infected by *P. chlamydosporia* and by the fact that the fungus expresses chitosanase and chitin deacetylase genes during egg infection. This suggests that these enzymes are important for the nematophagous activity of the fungus and they are targets for improving the capabilities of *P. chlamydosporia* as a biocontrol agent in agriculture.

Keywords: CAZymes - chitin deacetylases - chitosanases - nematode egg parasitism-nematophagous fungi - *Pochonia chlamydosporia*.

Introduction

Pochonia chlamydosporia (Goddard) Zare and Gams is a nematophagous fungus of the Hypocreales (Clavicipitaceae) belonging to Ascomycota. Since this fungus was reported as a parasite of cyst nematode eggs (Willcox and Tribe, 1974), it has been used as biocontrol agent to manage root-knot nematode (RKN) populations (Manzanilla-Lopez *et al.*, 2013). *P. chlamydosporia* can remain in the soil as a saprophyte (Domsch *et al.*, 1993) and can also colonize roots as a true endophyte (Maciá-Vicente *et al.*, 2009a; Escudero and Lopez-Llorca, 2012). Furthermore, *P. chlamydosporia* has been shown to promote growth in barley (Maciá-Vicente *et al.*, 2009b) and tomato (Zavala-Gonzalez *et al.*, 2015) plants. The multitrophic lifestyle of *P. chlamydosporia* (Larriba *et al.*, 2014) and its worldwide distribution make this fungus a good biocontrol agent for managing plant parasitic nematode (PPN) populations.

RKN females often remain embedded within galls and only a portion of their egg masses are exposed on the root surface (De Leij and Kerry, 1991). RKN are usually parthenogenetic organisms capable to reproduce with or without males. They have a short multiplication time which is even reduced with high soil temperatures (Maleita *et al.*, 2012). For these reasons, they are hard to control in the current climate change situation. The eggs are the most resistant life-stage of the nematodes in stressful environments (Curtis *et al.*, 2010). In *Meloidogyne javanica*, chitin represents 30%, and the protein matrix approximately 50% of the nematode eggshell (Curtis *et al.*, 2010). This structure is an important target for managing PPN diseases which cause large economic losses in agriculture (Stirling, 2014).

Several studies have demonstrated the importance of extracellular enzymes secreted by

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P. chlamydosporia during the egg-infection process (Huang *et al.*, 2004). *Pochonia* spp. proteases (Lopez-Llorca and Robertson, 1992; Segers *et al.*, 1995) and chitinases (Tikhonov *et al.*, 2002) can degrade key egg-shell components, and are therefore potential virulence factors during nematode infection. *P. chlamydosporia* secretes the serine protease VCP1 and a newly found serine carboxypeptidase (SCP1), when endophytically colonizing barley roots (Lopez-Llorca *et al.*, 2010). VCP1 and SCP1 have been also cloned, characterized and immunolocalized in *M. javanica* eggs infected by the fungus (Morton *et al.*, 2003; Larriba *et al.*, 2012; Escudero *et al.*, 2016). However, the mechanisms involved in the interaction between the fungus, the nematode and the plant root should be better understood in order to improve the performance of *P. chlamydosporia* as a biocontrol agent.

Chitosan is a highly deacetylated form of chitin (Rabea *et al.*, 2003) which is obtained after chitin deacetylation by chitin deacetylases or under alkaline conditions (Rinaudo, 2006). This polymer displays antimicrobial activity against bacteria (Liu *et al.*, 2004; Tikhonov *et al.*, 2006) and fungi (Palma-Guerrero *et al.*, 2008). However, entomopathogenic and nematophagous fungi as *P. chlamydosporia* are resistant to chitosan (Palma-Guerrero *et al.*, 2008) which increases their conidiation (Palma-Guerrero *et al.*, 2010). Previous studies have shown that chitosan induces both chitinolytic and chitosanolytic activities in *P. chlamydosporia*. On the contrary, chitin induces only chitinolytic activity of this fungus (Palma-Guerrero *et al.*, 2010). Collectively, these studies strongly suggest that chitosan metabolism could be another relevant player in *P. chlamydosporia* pathogenicity. However, there is only limited information about *P. chlamydosporia* chitosanases. The recent sequencing of *P. chlamydosporia* genome plus other -omics approaches provide new tools to improve our

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understanding of the ecology and pathogenicity of the fungus (Larriba *et al.*, 2014), including the identification of potential virulence factors.

The array of Carbohydrate-Active Enzymes (CAZymes) encoded by the genome of a fungus can often give insights into nutrient sensing and acquisition. We therefore conducted a targeted examination and comparison of the CAZymes in *P. chlamydosporia* to understand the multitrophic behavior of the fungus. Indeed the genome of *P. chlamydosporia* encodes putative enzymes for the breakdown of all plant cell wall polysaccharides, thereby explaining its endophytic lifestyle. Our analysis also reveals the importance of the metabolism of chitin and chitosan for *P. chlamydosporia* and its importance during nematode egg infection.

Results

The *P. chlamydosporia* genome encodes 292 glycoside hydrolases (GHs), a value higher than that of closely related fungal species (Table 1). To analyze how these GHs may contribute to the particular lifestyle of *P. chlamydosporia*, we examined the particular GH families known to be involved either with plant cell wall (PCW) polysaccharide breakdown and with chitin/chitosan degradation. We also investigated the occurrence of carbohydrate esterases (CEs) and carbohydrate binding modules (CBMs). Carbohydrate active enzymes with other roles are shown in Table S1 and their comparison with those encoded by other fungi is shown in Table S2. The distribution of overall number of CAZymes encoded in *P. chlamydosporia* presents significant differences respect to that of *Neurospora crassa*, *Arthrobotrys oligospora* and *Trichoderma virens* (Table S3).

The *P. chlamydosporia* genome encodes putative enzymes for plant cell wall

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degradation

Saprophytes, endophytes and plant pathogens have evolved particular arrays of CAZymes to utilize plant cell wall polysaccharides (cellulose and hemicellulose, pectin and xylan) as nutrients (Kubicek *et al.*, 2014). The *P. chlamydosporia* genome encodes a complete array of enzymes for the breakdown of plant cell wall polysaccharides. In this paper we follow the CAZyme family designations of the CAZy database (www.cazy.org; Cantarel *et al.*, 2009; Levasseur *et al.*, 2013; Lombard *et al.*, 2014).

Cellulose

Cellulose breakdown is achieved by the combined action of endoglucanases and cellobiohydrolases, assisted by lytic polysaccharide monooxygenases (LPMOs) (Medie *et al.*, 2012). Examination of the GHs families known to encode fungal cellobiohydrolases reveals that *P. chlamydosporia* encodes enzymes of both family GH6 and of family GH7 (Table 2), a similar situation to the saprotrophs examined, differently from the entomopathogens which have completely lost these enzymes that are essential for the breakdown of cellulose to soluble sugars (Table 2). *P. chlamydosporia* also encodes a GH45 endoglucanase (Table 2), a family also absent from entomopathogens except for *B. bassiana*. LPMOs are a recently identified family of enzymes that cleave polysaccharides such as cellulose or chitin with a copper-mediated oxidation (Harris *et al.*, 2010; Vaaje-Kolstad *et al.*, 2010; Quinlan *et al.*, 2011). Two main types of CBMs have been usually found appended to AA9 LPMOs, namely modules of families CBM1 and CBM18, which bind respectively cellulose and chitin suggesting the particular target of a putative LPMO. To analyze the potential of each fungal genome to oxidize cellulose, we have therefore restricted our analysis to LPMOs that had an appended

CBM1 module (Table 2). *P. chlamydosporia* and the saprotroph *N. crassa* encode,

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respectively, one and six CBM1-containing LPMOs of family AA9, while such enzymes are completely absent from the genomes of the entomopathogens *B. bassiana*, *C. militaris*, *M. acridum* and *M. anisopliae* (Table 2).

Xylan

Xylan, a heteropolysaccharide, is a major component of plant cell walls whose basic skeleton is a linear backbone of D-xylopyranose units connected by β -(1,4) glycosidic linkages (Bastawde, 1992) carrying various carbohydrate side chains and acetyl groups. Xylan breakdown requires endo- β -1,4-xylanases which are found in families GH10 and GH11, xylan α -glucuronidases from family GH67 and xylan acetyl esterases which are found in families CE1, CE3 and CE5 (along with cutinases for the latter family). Interestingly, the *P. chlamydosporia* genome encodes a member of each GH10 and GH11 families (Table 3), which are completely absent in the entomopathogens *C. militaris*, *M. anisopliae* and *M. acridum*, whereas *B. bassiana* encodes one GH10 enzyme (Table 3). Similarly, *P. chlamydosporia* has two GH67 members and this family is also absent from the entomopathogens (Table 3). Carbohydrate esterases are harder to analyze as their specificity for the polysaccharide is often rather low and they can cleave acetyl groups from various sources. However, *P. chlamydosporia* groups again with the saprotrophs with three CE1, two CE3 and seven CE5 members. Entomopathogenic fungi encode a few CE3 and CE5 members but less than *P. chlamydosporia*, *A. oligospora* or *Trichoderma* spp. genomes (Table 3). Overall these results show that the *P. chlamydosporia* genome still harbours a complete xylan degradation machinery, although not as developed as in *bona fide* saprophytic fungi.

Pectin

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Pectin is a structural heteropolysaccharide present in plant cell walls which mainly consists of esterified D-galacturonic acid residues α -1,4 linked. Various pectic polysaccharides can be detected in the plant cell wall, including homogalacturonan, xylogalacturonan, rhamnogalacturonans I and II, varying in content depending on the plant tissue and species (Harholt *et al.*, 2010). Conversely to cellulose and xylan breakdown examined above, the set of pectinolytic enzymes encoded by *P. chlamydosporia* is rather reduced with only three putative endopolygalacturonases of family GH28 (Table 4), and no pectin lyases (families PL1, PL4 and PL9) nor pectin esterases (families CE8 and CE12). This places *P. chlamydosporia* very close to *T. reesei*, which has only four GH28 enzymes, and not too far above entomopathogens which encode only one GH28. Only *A. oligospora* and *N. crassa* display a full pectinolytic apparatus (Table 4).

***P. chlamydosporia* enzymes for chitin and chitosan breakdown**

Chitin is the second most abundant natural polymer in the biosphere after cellulose. This homopolysaccharide made of N-acetylglucosamine residues joined by β -1,4 linkages occurs in nature as crystalline microfibrils in the exoskeleton of arthropods or in the cell walls of filamentous fungi and yeast (Rinaudo, 2006). Chitin breakdown by fungi is catalyzed by chitinases of family GH18 assisted by N-acetylglucosaminidases (GH20) and chitin-targeting LPMOs (family AA9 appended to CBM18 and family AA11). *P. chlamydosporia* genome encodes 24 GH18 chitinases (Table 5). This chitinolytic capacity is ca. two-fold that found for *Neurospora crassa* (13), higher than that for *Arthrobotrys oligospora* (17) and similar to that of entomopathogenic fungi such as *Beauveria bassiana* (22), *Cordyceps militaris* (23), *M. acridum* (19) and *M. anisopliae* (27). The largest GH18 producers are the mycoparasites such as *T. virens* (37) and *T.*

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atroviride (31) (Table 5), specialized in fungal cell wall degradation (Häkkinen *et al.*, 2012). The sole AA9 protein encoded by *C. militaris* carries the module of family CBM18 (data not shown), indicating that the most likely target for this enzyme is chitin. Although *P. chlamydosporia* genome does not encode any AA9 member appended with CBM18, this fungus contains 6 AA11 members (Table 5), which are LPMOs with chitinolytic activity (Hemsworth *et al.*, 2014).

Chitosan breakdown is an alternative pathway for chitin degradation (Hoell *et al.*, 2010). Chitosanases (EC 3.2.1.132) can be found in families GH5_2, GH7, GH8, GH46, GH75 and GH80 families, with the latter three families exclusively containing chitosanases. Our analyses showed that only GH75 family was found in the fungal genomes analyzed. GH75 chitosanases cleave chitosan in an endo-manner with an inverting mechanism (Shimosaka *et al.*, 1993; Cheng and Li, 2000). *P. chlamydosporia* genome encodes 11 putative GH75 chitosanases, a number particularly high (Table 5). We have designated these enzymes as Csn1-Csn11 (Table S1). Of all the GH75 members from *P. chlamydosporia* CAZome, only Csn4 contains a module of a polysaccharide lyase family 7 (GH75-PL7_4). *P. chlamydosporia* GH75 family expansion could be particularly relevant because the fungus is a parasite of nematode eggs whose eggshell is mainly composed of chitin and proteins (Bird and Bird, 1991). Other fungi phylogenetically closely related to *P. chlamydosporia* (Larriba *et al.*, 2014) such as *M. acridum*, *M. anisopliae*, *T. virens*, *T. reesei* and *T. atroviride* only encode 3, 3, 5, 3 and 6 chitosanases, respectively (Table 5). Significant differences were found between the distribution of CAZymes targeting chitin and chitosan encoded in *P. chlamydosporia* genome respect to *A. oligospora*, *C. militaris* and *T. virens* (Table S3).

***P. chlamydosporia* genome shows a large and recent expansion of family GH75 chitosanases**

Birth and death analysis revealed the evolution of the GH75 gene number among the 34 fungal species tested. Figure 1 shows the pattern of expansion of family GH75 with the estimated number of GH75 genes at the different nodes in the phylogenetic tree. An expansion of the number of GH75 genes occurred in the common ancestor of *Trichoderma* spp., *Metarhizium* spp. and *P. chlamydosporia* which had three genes (Fig. 1). While a moderate subsequent expansion is visible in members of the *Trichoderma* clade (reaching 6 copies in *T. atroviride*), *P. chlamydosporia* has seen its number of GH75 grow to 11 copies after it separated from *Metarhizium* spp. This recent and massive expansion potentially provides *P. chlamydosporia* with an abundant enzymatic machinery for chitosan degradation. Interestingly *P. chlamydosporia* displayed three closely-related paralogues (Csn3, Csn4 and Csn5), confirming that the gene expansion is due to recent duplication events (Fig. 2).

***P. chlamydosporia* displays high efficiency in using chitosan as growth substrate**

In order to check the chitin and chitosan-degrading capabilities of *P. chlamydosporia*, *B. bassiana*, *M. anisopliae* and *N. crassa*, we evaluated their growth in liquid media supplemented with colloidal chitin and chitosan (Fig. 3). We also tested glucose (0.5%) and PDB (6 g l⁻¹) as positive controls to compare fungal growth using different nutritional sources. *P. chlamydosporia* was, by far, the most capable fungus to use chitosan as a growth substrate. This polymer appears to be used more efficiently as substrate by *P. chlamydosporia* than glucose and chitin. In fact, fungal growth is ca. four-fold and ca. two-fold in chitosan respect to glucose and chitin, respectively (Fig.

3A). The entomopathogens *M. anisopliae* and *B. bassiana* followed *P. chlamydosporia*,

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but their growth 20 days after inoculation was approximately one third of that attained by *P. chlamydosporia* (Fig. 3A-D). Chitosan virtually arrested *N. crassa* growth (Fig. 3D). It is worth mentioning that unlike chitin, chitosan caused a lag phase (initial growth inhibition) for all fungi tested. The lag phase was of only 4 days for *P. chlamydosporia* and *M. anisopliae*, and of 8 and 16 days for *B. bassiana* and *N. crassa*, respectively (Fig. 3A-D). Both *M. anisopliae* and *B. bassiana* can use chitin and chitosan as substrates but less efficiently than PDB (Fig. 3B-C). On chitin, *B. bassiana* was the fastest growing fungus, followed by *P. chlamydosporia* and *M. anisopliae* (Fig. 3C). On the other hand *N. crassa* displayed a very limited capacity to grow on chitin (Fig. 3D).

Chitosan is produced in nematode eggs infected by *P. chlamydosporia*

To investigate the functional role of the expanded family GH75 in *P. chlamydosporia*, we analyzed the presence of chitosan in *P. chlamydosporia* mycelia, root-knot nematode *M. javanica* eggs and in nematode eggs infected by *P. chlamydosporia*. Figure 4 shows differential interference contrast (DIC) images (A, D, G and J), fluorescence images for eggs autofluorescence (550-620 nm; B, E, H, K) and FITC detection (500-520 nm; C, F, I, L) for chitosan signal. Chitosan was only weakly detected in *P. chlamydosporia* germ tubes (Fig. 4A-C, arrowheads) and was not found in *M. javanica* eggshell (Fig. 4D-I). By contrast, chitosan labelling was high in areas of egg penetration by *P. chlamydosporia* (Fig. 4J-L, arrowheads). These results suggest that chitosan could be produced by the chitin deacetylases (CE4) from *P. chlamydosporia* during the infection process.

***P. chlamydosporia* chitosanase and chitin deacetylase genes are induced when**

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infecting nematode eggs

We quantified by qPCR the expression of several members of *P. chlamydosporia* GH75 gene family (Csn1, Csn3, Csn4, Csn5, Csn6, Csn7, Csn8, Csn9, Csn10 and Csn11) (Fig. 5). Interestingly, eight of these chitosanases displayed higher levels of expression *csn3* (six-fold), *csn4* (five-fold), *csn5* (four-fold), *csn7* (four-fold), *csn8* (six-fold), *csn10* (ca. three-fold), when *P. chlamydosporia* parasitizes nematode eggs than with *P. chlamydosporia* alone, specially *csn1* (fifty-fold) and *csn6* (seventy-fold); (Fig. 5A-I). Only *csn9* gene did not show a difference in expression (Fig. 5H). We could not detect *csn11* expression under our experimental conditions (*P. chlamydosporia* or *P. chlamydosporia* with *M. javanica* eggs). Conventional PCR confirmed the correct gene amplification (Fig. S1).

In addition, we also analyzed the expression of Cda1 and Cda2 chitin deacetylases (both CDAs belong to the CE4 family) under the same conditions. *P. chlamydosporia cda1* and *cda2* genes were also induced (ca. thirteen-fold and ca. three-fold, respectively) when the fungus infects *M. incognita* eggs (Fig. 6A-B). After PCR amplification (Fig. S2), no differences on the amplicon size band between gDNA and cDNA were expected for these *P. chlamydosporia* CDAs (CE4) because their CE4 domains gene sequences do not contain any introns. Our results indicate that *P. chlamydosporia* expresses chitosanases and chitin deacetylases encoding genes during RKN egg infection.

Discussion

Although the presence of genes is not indicative of protein functionality, several studies have demonstrated a correlation between the repertoire of CAZymes in fungal genomes and their lifestyles and carbon acquisition modes (Kohler *et al.*, 2015; Riley *et al.*, 2014). Our detailed analysis of the enzymes that breakdown polysaccharides encoded

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by the genome of *P. chlamydosporia* reveals notable differences with phylogenetically close fungi such as the entomopathogens *M. anisopliae* or *M. acridum*.

Larriba *et al.*, (2014) reported the presence of a high number of hydrolase encoding genes in *P. chlamydosporia* genome. This study included a functional analysis of gene expression during barley root endophytic colonization by *P. chlamydosporia* but CAZy families were not analysed in detail. They concluded that GHs and CEs accounted for more than 40% of the genes expressed. Our results support these data indicating that *P. chlamydosporia* CAZome has the tools to deconstruct the main PCW polysaccharides components such as cellulose, xylan and pectin as nutritional substrates (De Vries and Visser, 2001; King *et al.*, 2011). The corresponding CAZymes could also play an important role in the endophytic lifestyle of the fungus (Maciá-Vicente *et al.*, 2009a; Escudero and Lopez-Llorca, 2012; Larriba *et al.*, 2015). Comparison to other nematophagous fungi shows that *A. oligospora* (which uses traps instead of appressoria) has a large arsenal of enzymes for the degradation of PCW polysaccharides. *A. oligospora*, unlike *P. chlamydosporia*, causes necrosis in barley roots (Bordallo *et al.*, 2002). In advanced stages of root colonization, the former fungus was even able to decorticate areas of the root. We speculate that this more aggressive behavior could be related to the much larger amount of pectin degrading enzymes in *A. oligospora* compared to *P. chlamydosporia*. These enzymes loosen cells in plant tissues by cleaving the pectin molecules present in the middle lamella holding adjacent cells together. The number of *P. chlamydosporia* CAZymes involved in PCW degradation is reduced compared to that found in *A. oligospora* or *N. crassa*. However, *P. chlamydosporia* has retained a rather complete PCW degrading machinery, unlike the closely related entomopathogens, *M. anisopliae* and *M. acridum*. The CAZymes encoded by these

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fungi are directed to insect chitin degradation according to their entomopathogenic lifestyle (Samuels *et al.*, 1989). Since cyst and root-knot nematode female and eggs which are embedded in plant root tissues are the main nutritional resource of *P. chlamydosporia* (Lopez-Llorca and Duncan, 1991), we hypothesize that the fungus has retained PCW degrading enzymes to survive in its particular niche. By contrast, most targets of entomopathogenic fungi are not deeply associated with plant tissues in their lifecycles. Consequently, entomopathogen genomes have lost most CAZyme families required for plant cell wall polysaccharide degradation (Xiao *et al.*, 2012).

The extracellular enzymes secreted by *P. chlamydosporia* are relevant for degrading the nematode eggshell during infection (Yang *et al.*, 2007). This structure is the first barrier against penetration by the fungus and it is mainly composed of chitin fibers in a protein matrix (Bird and McClure, 1976; Bird and Bird, 1991). The *P. chlamydosporia* CAZyme arsenal shows a high number of enzymes for chitin breakdown. This is in accordance with its high specialization in nematode eggs parasitism using chitinases (Tikhonov *et al.*, 2002; Mi *et al.*, 2010). Furthermore, Mi *et al.*, (2010) suggested that chitinases from *P. chlamydosporia* could also be involved in insect cuticle damage. Other nematophagous fungi such as *A. oligospora*, use extracellular enzymes to degrade nematode cuticles (Tunlid and Janson, 1991; Yang *et al.*, 2013) as well as nematotoxins (Olthof and Estey, 1963) involved in its trapping nematophagous lifestyle (Nordbring-Hertz, 2004). The CAZymes encoded in *A. oligospora* genome reveal that this fungus not only has kept a complete PCW degradation machinery, but also has a chitinolytic degradation arsenal, though less numerous than the *P. chlamydosporia* chitin modifying enzymes. Unlike *A. oligospora*, the analysis of the CAZyme profile of *P. chlamydosporia* also reveals a large and recent expansion of family GH75 chitosanases.

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The presence of 11 GH75 members could explain the excellent ability of this fungus to degrade chitosan and use this polymer as a nutrient for growth compared to the other fungi tested and that have a smaller number of members for this family. Our results provide evidence about *P. chlamydosporia* can use chitosan as nutritional source similar to a typical fungal culture medium (PDB). This observation may also provide an explanation as to why *P. chlamydosporia* is resistant to this polymer while other fungi show an arrested growth in the presence of chitosan (Palma-Guerrero *et al.*, 2008). We also show a phylogenomic analysis of *P. chlamydosporia* GH75 family, providing data on the evolution of these enzymes which could be involved in the *P. chlamydosporia* biology.

Unlike chitin, which is a polymer widely present in nature (Rinaudo *et al.*, 2006), chitosan is only produced after chitin deacetylation under acidic conditions (Zhao *et al.*, 2010). Our immunolabelling results show chitosan production by *P. chlamydosporia* during RKN egg infection, mostly in appressoria. Differentiation of appresoria is carried out by *P. chlamydosporia* during nematode egg infection (Lopez-Llorca and Claugher, 1990). These structures are well known areas in fungi for enhanced secretion of extracellular enzymes such as proteases (Leger *et al.*, 1991; Lopez-Llorca and Robertson, 1992). Previous reports have described changes in germ tubes and appressoria fungal cell wall during the penetration to the host plant (Fujikawa *et al.*, 2009). In fact, fungal plant pathogens seem to deacetylate chitin from their own cell wall in order to avoid the effect of chitinases secreted as plant immune responses (El Gueddari *et al.*, 2002; Kouzai *et al.*, 2012). In our results, we have detected chitosan in the fungal cell wall of *P. chlamydosporia* indicating that the fungus is also able to deacetylate chitin from its own cell wall. Since *P. chlamydosporia* is a root endophyte in

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both mono and dicots (Bordallo *et al.*, 2002; Maciá-Vicente 2009b; Escudero and Lopez-Llorca, 2012), the role of *P. chlamydosporia* CDAs to avoid chitinases of the plant immune system should be evaluated in future studies. However, we have also detected an intense chitosan immunolabelling associated with the *P. chlamydosporia* appressoria suggesting the capacity of the fungus to deacetylate nematode egg chitin during penetration. The resulting chitosan could be synergistically depolymerized by the expanded family of chitosanases encoded by the *P. chlamydosporia* genome and thus explain the ability of this fungus to parasitize nematode females and eggs (De Leij and Kerry, 1991). Taking in account the fact that chitin induces chitinases while chitosan induces both chitinases and chitosanases (Palma-Guerrero *et al.*, 2010), we would propose that CDAs activity, producing chitosan, induces chitosanases (and also chitinases) during the RKN egg infection process.

Despite the fact that most studied extracellular enzymes secreted by nematophagous fungi as virulence factors have been proteases and chitinases (Tikhonov *et al.*, 2002; Huang *et al.*, 2004; Yang *et al.*, 2007; Lopez-Llorca *et al.*, 2010; Mi *et al.*, 2010; Larriba *et al.*, 2012; Escudero *et al.*, 2016), our results clearly show that *P. chlamydosporia* widely induces chitosanases and chitin deacetylases genes during the infection of nematode eggs. Specifically, the high fold induction of the gene expression of chitosanases Csn1 and Csn6 by the presence of nematode eggs suggests these genes as candidates to be involved in PPN eggs parasitism. However, massive transcriptomic (RNAseq) would be useful to improve our knowledge about *P. chlamydosporia* gene expression during pathogenetic process. Interestingly, despite having increased expression in the presence of *M. javanica* eggs, the three closely-related paralogues (Csn3, Csn4 and Csn5) are not highly expressed under these conditions. This may

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indicate different functionality or environmental requirements for expression of these genes. Previous studies have shown chitosanase expression related with *Fusarium solani* pathogenicity (Liu *et al.*, 2010) and the essentiality of CDAs (and therefore chitosan) for pathogenesis in *Cryptococcus* spp. (Baker *et al.*, 2007). In our study, *P. chlamydosporia* appears to deacetylate chitin from nematode egg-shell, producing chitosan which can then be degraded with the large array of GH75 chitosanases of the fungus. Previous results have indicated the dual function of chitin deacetylases modifying the fungal cell wall and being involved in the degradation of insect cuticle by *M. anisopliae* (Nahar *et al.*, 2004). Thus fungal N-deacetylation of chitin to chitosan probably represents an undiscovered –perhaps early– step in nematode egg-infection by *P. chlamydosporia*. The detailed analysis of the CAZyme portfolio encoded by the genome of *P. chlamydosporia* together with our expression analysis in the presence of RKN eggs reveals the importance of uncharacterized enzymes encoded this fungus. Further studies will exploit these new enzymes and their regulation for the improvement of *P. chlamydosporia* as a biological control agent against PPN.

Experimental procedures

CAZyme annotation

A total of 11,079 protein-encoding ORFs from the *P. chlamydosporia* genome were analyzed using the tools used for updating the Carbohydrate-active enzymes database (CAZy: www.cazy.org; Cantarel *et al.*, 2009; Levasseur *et al.*, 2013; Lombard *et al.*, 2014). Briefly, the identification of CAZymes involved a Blastp comparison against the full length sequences from the CAZy database. This comparison was followed by a modular annotation procedure which identifies constitutive modules on the peptide using hits against libraries of isolated catalytic and carbohydrate-binding modules using

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both BlastP and Hidden Markov profile models. Annotation results were curated manually to eliminate or correct artifacts (e.g. fragments from sequencing or splicing variants). The *P. chlamydosporia* set of CAZymes was compared to that of phylogenetically close fungi (*Metarhizium anisopliae*, *M. acridum*, *Cordyceps militaris* and *Beauveria bassiana*). These fungal parasites of insects are being used as biological control agents (Zimmermann, 1993; Ricaño *et al.*, 2013; Priwiratama and Susanto, 2014). The comparison was extended to the mycoparasites *Trichoderma virens* and *T. atroviride*. *Trichoderma spp.* capable of degrading both fungal and plant cell wall material (Häkkinen *et al.*, 2012). We also compared with *T. reesei* a different *Trichoderma* species which presents a saprophytic lifestyle (Druzhinina, *et al.* 2011). Finally, the arrays of CAZymes encoded by the nematode-trapping fungus *Arthrobotrys oligospora* and by the saprotroph *Neurospora crassa* were also included in our analyses. Annotation results were statistically analyzed using Chi-squared Test of Independence for the comparison of *P. chlamydosporia* CAZymes distribution with that of other fungi for each table separately (p-value 0.05). The analysis was performed using R version 3.0.2. Accession numbers for fungal genomes compared appear in Table S4.

Expansion of the number of GH75 genes during evolution

To evaluate the expansion of the number of GH75 genes in fungal genomes a phylogenetic tree of 34 fungi was analyzed using a stochastic birth and death model with the CAFE software (De Bie *et al.*, 2006). All the selected genomes are public and the phylogenetic tree was constructed using the concatenation of the following six single copy and well-conserved proteins present in these fungal genomes: Phosphoglycerate kinase, DNA-directed RNA-polymerase II subunit RPB1, calmodulin, glyceraldehyde-3-phosphate dehydrogenase (GADPH), DNA-directed

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RNA polymerase II subunit RPB2 and the Translation elongation factor 1 alpha (TEF1 α). These sequences were aligned with Muscle (Edgar, 2004) and the phylogenetic tree (Fig. S3) was constructed using the Maximum Likelihood method with bootstrap (500 replicates) and the LG model as the substitution model (Le and Gascuel, 2008) implemented in the MEGA6.06 software (Tamura *et al.*, 2013). The number of GH75 genes in 34 fungal genomes is reported in Table S5. A phylogenetic tree was also constructed as above using the fungal species in the CAZome comparison (Tables 1-5).

Phylogenetic analysis of family GH75

The 11 GH75 sequences from *P. chlamydosporia* were aligned with 66 GH75 sequences from 33 other fungal genomes from the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov>) and JGI Genome Portal database (<http://genome.jgi.doe.gov>) using Muscle (Edgar, 2004). A cladogram was then calculated using the Neighbour-Joining method (Saitou and Nei, 1987) and JTT model (Jones, Taylor and Thornton, 1992) with bootstrap values calculated using 1000 replicates implemented in the MEGA6.06 software (Tamura *et al.*, 2013). The cladogram was displayed using Dendroscope (Huson and Scornavaca, 2012). The five following GH75 sequences were removed before cladogram building because they were fragmentary and/or affected by large splicing issues: *Thozetella* sp. (ThoPMI491_1|264981), *Chaetomium globosum* (XP_001223450.1), *Trichoderma atroviride* (EHK46099.1), *Metarhizium acridum* (XP_007816114.1) and *Colletotrichum graminicola* (EFQ35368.1).

Strains and growth conditions

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Fungal strains used in this work were the nematophagous fungus *Pochonia chlamydosporia* Pc123 (ATCC No. MYA-4875; CECT No. 20929) isolated from naturally infected nematode eggs (Olivares-Bernabeu and Lopez-Llorca, 2002) and the entomopathogenic fungi, *Metarhizium anisopliae* isolate 46 (CECT No. 20928) from *Otiorynchus sulcatus* and *Beauveria bassiana* isolate 53 (CECT No. 20927) from *Rhizotrogus chevrolatti*. The saprophytic fungus *Neurospora crassa* wild-type strain 74-OR23-IVA (FGSC #2489) was also used. Fungi were maintained on Corn Meal Agar (CMA; Becton Dickinson) except for *N. crassa* which was maintained on Vogel's solid medium (Vogel, 1956) supplemented with 2% sucrose and 1.5% technical agar (Cultimed).

Fungal growth on chitin and chitosan

Conidia from *P. chlamydosporia*, *M. anisopliae*, *B. bassiana* and *N. crassa* were obtained from 20-day-old colonies on CMA (Vogel's medium for *N. crassa*) using 0.02% Tween-20 in sterile distilled water. Resulting conidia suspensions were then counted in a haemocytometer. Ninety-six-well Microtiter plates (Sterilin) with 200 μ l per well of either at 0.5% (w/v) colloidal chitin or chitosan were inoculated with $2 \cdot 10^5$ conidia per well. Chitosan with a molecular weight of 70 kDa and exhibiting 80.5% deacetylation degree was obtained from Marine BioProducts GmbH (Bremerhaven, Germany). It was prepared as described in Palma-Guerrero *et al.* (2010). Briefly, it was dissolved in 0.25M HCl and pH adjusted to 5.6 with 1M NaOH. The resulting solution was dialyzed for salt removal and then autoclaved at 120 °C for 20 min. Colloidal chitin (Sigma, St. Louis, MO, USA) was prepared with 37% HCl at 4 °C for 16 h with agitation, followed by 50% ethanol precipitation and distilled water rinsing until pH value reached 5.6. The gel obtained was passed through 0.5 and 0.25 mm mesh size

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sieves and resuspended in distilled water at a final concentration of 10 mg ml⁻¹. Glucose 0.5% (w/v) and 6 g l⁻¹ Potato Dextrose Broth (PDB, Becton Dickinson) were used as controls to compare chitin and chitosan with other carbon sources. Distilled water was used as negative control (no nutrients). Fungal growth was estimated by daily recording (OD₄₉₀) in a GeniosTM Multiwell Spectrophotometer (Tecan Männedorf, Switzerland) for 20 days (Lopez-Moya *et al.*, 2015).

Chitosan immunolocalization on *P. chlamydosporia* germlings and *M. javanica* infected eggs

P. chlamydosporia conidia were collected from two-week-old CMA cultures in 5 ml sterile distilled water and filtered through Miracloth (Calbiochem) to remove hyphae.

RKN (*Meloidogyne javanica*) egg masses dissected from infested tomato roots (cv. Marmande) were hand-picked, surface-sterilized as in Escudero and Lopez-Llorca (2012) and kept at 4°C until used. Aliquots (60µl) each containing ca. 10 eggs were inoculated with ca. 10⁴ *P. chlamydosporia* conidia per egg aliquot and incubated for 5 days at 25 °C on Superfrost slides (Thermo Scientific) then air-dried. *P. chlamydosporia* germlings were obtained from conidial suspensions at 10⁴ conidia µl⁻¹ and nematode egg aliquots (each containing ca. 10 eggs) were incubated for 24 h at 25 °C and air-dried too. Chitosan immunolabelling was carried out as in Thornton and Talbot (2006).

Briefly, dried samples were incubated for 1 h at 23°C with 60µl of blocking buffer (10% Goat Serum in PBS). Slides were washed three times (5 min each) with PBS (0.8% NaCl, 0.02% KCl, 0.115% Na₂HPO₄, 0.02% KH₂PO₄, pH=7.2) and then incubated for 2 h with 40 µg ml⁻¹ mAbG7 an anti-chitosan monoclonal antibody in PBS (Schubert *et al.*, 2010). Slides were washed three times (5 min each) with PBS and then incubated

for a further 1 h with goat-anti-mouse polyvalent FITC conjugate (Sigma) diluted 1:40

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in blocking buffer. Slides were washed under agitation (80 rpm) three times (5 min each) with PBS and were sealed with coverslips using Fluoromount (Sigma). All incubation steps were performed at room temperature in a moist chamber. Fluorescence of samples was visualized using a Leica TCS-SP2 laser-scanning confocal microscope. Samples were excited with a 488 nm laser, FITC fluorescence detected at 500-520 nm and egg autofluorescence was tested at 550-620 nm (Escudero and Lopez-Llorca, 2012). Negative controls (omission of primary or secondary antibodies) were also included (Fig. S4). A 2 μ l dot blot assay was carried out to test mAbG7 monospecificity using colloidal chitin (Sigma), chitosan (Marine Bioproducts; 70 kDa) prepared as described above, chitooligosaccharide (COS; 5,44 kDa), N-acetylglucosamine (Sigma), glucosamine (Sigma), soluble starch (Sigma). All compounds were tested at both 1 and 10 μ g ml⁻¹. A *M. javanica* egg aliquot (10 egg μ l⁻¹) was also included for checking the absence of chitosan in nematode eggs and distilled water and PBS as negative controls (Fig. S5).

DNA and RNA isolation

P. chlamydosporia was inoculated in 100 ml flasks each containing Czapek Dox broth medium (NaNO₃ 3 g, KCl 0.5 g, magnesium glycerophosphate 0.5g, FeSO₄ 0.01 g, K₂SO₄ 0.35 g, sucrose 30 g, 0.5 g yeast extract, pH 6.8) l⁻¹ (Ward *et al.*, 2012) for fungal growth and incubated at 25°C for 5 days with shaking at 120 rpm. The mycelium was then collected by filtration through Miracloth (Calbiochem) and washed twice with sterile distilled water. The resulting mycelium (0.2 g) was inoculated in either minimal medium (MM; Sucrose 1 mg, NaNO₃ 14 mg, MgSO₄ 0.25 g, KCl 0.25 g, K₂HPO₄ 0.5g, FeSO₄ 0.06 g, pH 6.8) l⁻¹ or MM amended with *Meloidogyne javanica* eggs (1 egg μ l⁻¹) and then incubated for 4 days at 25°C under shaking at 120 rpm. Mycelia were finally

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collected by filtration, frozen in liquid N₂, lyophilized and stored at -80 °C until used. Surface sterilized *M. javanica* eggs were used as negative controls. Total DNA was extracted from ca. 0.2 g of lyophilized samples using a cetyl-trimethyl-ammonium bromide (CTAB)-based extraction method adapted from O'Donnell *et al.*, (1998). Total RNA was extracted using TRIzol reagent (Life Tech) according to the manufacturer's instructions from ca. 0.2 g lyophilized material. All RNA samples were subjected to electrophoresis on 2% agarose gels stained with ethidium bromide (Sigma) before DNase treatment to detect RNA presence (Fig. S6).

PCR and RT-PCR (qPCR)

Specific primers (Table S6) were designed using Primer3 (Untergasser *et al.*, 2012) and evaluated with NetPrimer software (www.premierbiosoft.com) to flank one intron of ten *P. chlamydosporia* chitosanases (except for *csn2* gene which was predicted as intracellular chitosanase; Larriba *et al.*, 2014) and two chitin deacetylase encoding genes (CE4 family; *cdal* and *cda2*), all containing a predicted signal peptide. They were identified in Larriba *et al.*, (2014) using SignalP (<http://www.cbs.dtu.dk/services/SignalP>). *P. chlamydosporia* gene sequences (Larriba *et al.*, 2014) were obtained from www.fungalinteractions.org (accession date 5 February 2015). Csn1, Csn3, Csn4, Csn5, Csn6, Csn7, Csn8, Csn9, Csn10 and Csn11 chitosanases correspond to Pc_6893, Pc_6897, Pc_6900, Pc_6891, Pc_6892, Pc_6896, Pc_6899, Pc_6895, Pc_6894 and Pc_7775 respectively, while Cda1 and Cda2 chitin deacetylases correspond respectively to Pc_2565 and Pc_2566 *P. chlamydosporia* gene sequences (Table S1).

PCR reactions were carried out using Taq 2X Master Mix Red (VWR), 50 ng of template (gDNA or cDNA) and forward and reverse primers. After an initial

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denaturation step at 95° C for 5 min, amplification was performed for 35 cycles each of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, followed by a final step at 95°C for 3 min. PCR products were subjected to electrophoresis on 2% agarose gels stained with Gel Red (VWR).

RNA samples for RT-PCR were treated with DNase (Turbo DNA-free, Ambion) to remove DNA remains and a PCR was performed on RNA samples using primers for β -tubulin gene (Ward *et al.*, 2012) to check out absence of gDNA. cDNA was then synthesized using RNA (1 μ g per sample), retrotranscriptase RevertAid (Thermo) and oligo dT (Thermo). Differences on the band size between gDNA and cDNA indicated the effectiveness of retrotranscription and ruled out gDNA contamination. For gene expression quantification, 1 μ l of the cDNA was used as template in each qPCR reaction. Relative changes in gene expression levels were determined using the Step One Plus system (Applied Biosystem) with the FastStart Universal SYBR Green Master amplification kit according to the manufacturer's instructions (Roche Diagnostics). Genes for allantoate permease (Rosso *et al.* 2014), glyceraldehyde 3-phosphate dehydrogenase (Escudero *et al.*, 2016) and β -tubulin (Ward *et al.* 2012), were used as housekeeping genes (Table S4). After expression analysis, RNA levels were normalized to the constitutively expressed gene β -tubulin (Ward *et al.*, 2012) in *P. chlamydosporia*. Each experiment was repeated twice and each reaction was performed in triplicate. Statistical significance was estimated by the Student's t-test according to previous description (Pfaffl, 2001).

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TABLES AND FIGURE LEGENDS

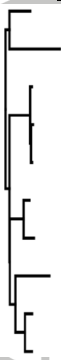
Table 1. Overall number of degradative carbohydrate-active enzymes in broad classes (GH, glycoside hydrolases; PL, polysaccharide lyases; CE carbohydrate esterases; CBM, carbohydrate-binding modules; AA, auxiliary activities) for fungi with diverse lifestyles. Abbreviations: S (Saprophyte), E (Entomopathogen), N (Nematophagous), M (Mycoparasite). Figure on the left of the table represents the phylogenetic relationship between fungal species. Grey background indicates the fungi with similar distribution of CAZymes to *P. chlamydosporia*.



Fungus	Lifestyle	GH	PL	CE	CBM	AA	Total
<i>Neurospora crassa</i>	S	192	4	24	71	54	345
<i>Arthrotrichum oligospora</i>	N	202	15	29	179	51	476
<i>Metarhizium acridum</i>	E	175	3	10	24	43	255
<i>Pochonia chlamydosporia</i>	N	292	7	17	69	54	439
<i>Metarhizium anisopliae</i>	E	192	3	10	52	55	312
<i>Beauveria bassiana</i>	E	174	2	15	66	41	298
<i>Cordyceps militaris</i>	E	162	3	13	66	38	282
<i>Trichoderma atroviride</i>	M	255	8	22	92	36	413
<i>Trichoderma reesei</i>	S	200	5	16	58	33	312
<i>Trichoderma virens</i>	M	269	6	24	111	41	451

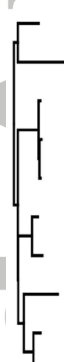
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Table 2. Cellulose targeting CAZymes encoded by *P. chlamydosporia* and other fungal genomes. Abbreviations: S (Saprophyte), E (Entomopathogen), N (Nematophagous), M (Mycoparasite). The family and subfamily designations follow the conventions of the CAZy database (www.cazy.org). Figure on the left of the table represents the phylogenetic relationship between fungal species.



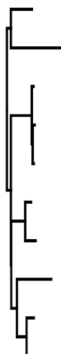
Fungus	Lifestyle	Families targeting cellulose				
		GH5_5	GH6	GH7	GH45	AA9 - CBM1
<i>Neurospora crassa</i>	S	1	3	5	1	6
<i>Arthrobotrys oligospora</i>	N	5	2	6	4	14
<i>Metarhizium acridum</i>	E	1	0	0	0	0
<i>Pochonia chlamydosporia</i>	N	2	1	1	1	1
<i>Metarhizium anisopliae</i>	E	1	0	0	0	0
<i>Beauveria bassiana</i>	E	1	0	0	1	0
<i>Cordyceps militaris</i>	E	1	0	0	0	0
<i>Trichoderma atroviride</i>	M	3	1	2	1	1
<i>Trichoderma reesei</i>	S	2	1	2	1	1
<i>Trichoderma virens</i>	M	3	1	2	2	1

Table 3. Xylan-targeting CAZymes encoded by *P. chlamydosporia* and other fungal genomes. Abbreviations: S (Saprophyte), E (Entomopathogen), N (Nematophagous), M (Mycoparasite). The family designations follow the conventions of the CAZy database (www.cazy.org). Figure on the left of the table represents the phylogenetic relationship between fungal species.




Fungus	Lifestyle	Families targeting xylan						
		GH10	GH11	GH67	GH74	CE1	CE3	CE5
<i>Neurospora crassa</i>	S	4	2	1	1	6	3	3
<i>Arthrobotrys oligospora</i>	N	4	3	0	3	8	0	4
<i>Metarhizium acridum</i>	E	0	0	0	0	2	1	2
<i>Pochonia chlamydosporia</i>	N	1	1	2	0	3	2	7
<i>Metarhizium anisopliae</i>	E	0	0	0	0	1	1	2
<i>Beauveria bassiana</i>	E	1	0	0	0	3	1	5
<i>Cordyceps militaris</i>	E	0	0	0	0	3	1	4
<i>Trichoderma atroviride</i>	M	1	4	2	1	3	4	5
<i>Trichoderma reesei</i>	S	1	3	1	1	2	3	4
<i>Trichoderma virens</i>	M	2	4	2	1	4	3	6

Table 4. Pectin-targeting CAZymes encoded by *P. chlamydosporia* and other fungal genomes. Abbreviations: S (Saprophyte), E (Entomopathogen), N (Nematophagous), M (Mycoparasite). The family designations follow the conventions of the CAZy database (www.cazy.org). Figure on the left of the table represents the phylogenetic relationship between fungal species.



Fungus	Lifestyle	Families targeting pectin					
		GH28	CE8	CE12	PL1	PL3	PL4
<i>Neurospora crassa</i>	S	2	2	1	1	1	1
<i>Arthrobotrys oligospora</i>	N	7	5	3	8	1	1
<i>Metarhizium acridum</i>	E	1	0	0	0	0	0
<i>Pochonia chlamydosporia</i>	N	3	0	0	0	0	0
<i>Metarhizium anisopliae</i>	E	1	0	0	0	0	0
<i>Beauveria bassiana</i>	E	1	0	0	0	0	0
<i>Cordyceps militaris</i>	E	1	0	0	0	0	0
<i>Trichoderma atroviride</i>	M	6	1	0	2	0	0
<i>Trichoderma reesei</i>	S	4	0	0	0	0	0
<i>Trichoderma virens</i>	M	6	2	0	0	0	0

Table 5. CAZyme families targeting chitin and chitosan encoded by *P. chlamydosporia* and other fungal genomes. Abbreviations: S (Saprophyte), E (Entomopathogen), N (Nematophagous), M (Mycoparasite), CDAs (Chitin deacetylases). The family designations follow the conventions of the CAZy database (www.cazy.org). Figure on the left of the table represents the phylogenetic relationship between fungal species. Grey background indicates the fungi with similar distribution respect to *P. chlamydosporia* of CAZymes related to chitin and chitosan modification.



Fungus	Lifestyle	Chitin						Chitosan	CDAs
		GH18	GH20	CBM18	CBM50	AA9-CBM18	AA11	GH75	CE4
<i>Neurospora crassa</i>	S	13	2	9	18	0	4	1	5
<i>Arthrobotrys oligospora</i>	N	17	1	18	39	0	3	1	0
<i>Metarhizium acridum</i>	E	19	2	3	5	0	5	3	2
<i>Pochonia chlamydosporia</i>	N	24	8	10	18	0	6	11	3
<i>Metarhizium anisopliae</i>	E	27	2	8	26	0	7	3	3
<i>Beauveria bassiana</i>	E	22	6	15	26	0	4	2	3
<i>Cordyceps militaris</i>	E	23	3	17	33	1	4	2	3
<i>Trichoderma atroviride</i>	M	30	3	21	24	0	2	6	4
<i>Trichoderma reesei</i>	S	20	3	8	17	0	3	3	3
<i>Trichoderma virens</i>	M	37	3	23	40	0	4	5	4

Figure 1. Evolutionary gains and losses in family GH75 chitosanases.

Phylogenetic tree was calculated using Maximum likelihood method and LG substitution model in MEGA6.06 software and shows the evolutionary relation between 34 fungi and the gain (red), losses (blue) and remained (black) GH75 members into their genomes. The number in the nodes is the estimated number of members in the common ancestor and the number on the branch shows the expansion or contraction size. Bootstrap values are indicated in Fig. S3. Bars represent the number of GH75 enzymes encoded for each fungus genome.

Figure 2. Evolutionary relatedness of *P. chlamydosporia* GH75 chitosanases.

The cladogram displaying branching of 77 fungal GH75 sequences from 34 fungal genomes. GH75 members from *P. chlamydosporia* genome appear in red. The accession numbers for each sequence is indicated on the branches.

Figure 3. Colloidal chitin and chitosan as nutritional substrates.

Fungal growth of *P. chlamydosporia* (A), *M. anisopliae* (B), *B. bassiana* (C) and *N. crassa* (D) 0.5% colloidal chitin and 0.5% chitosan. Glucose (0.5%) and PDB (6g l⁻¹) were used as positive controls while water was used as negative control (no nutrients).

Figure 4. Chitosan immunolocalization.

Chitosan immunolocalization on *P. chlamydosporia* mycelium (A-C), on *M. javanica* eggs in two different developmental stages (D-F and G-I, respectively) and in *M. javanica* eggs parasited by *P. chlamydosporia* (J-L). (DIC) images (A, D, G and J), fluorescence images for eggs autofluorescence (550-620 nm; B, E, H, K) and FITC detection (500-520 nm; C, F, I, L) show chitosan production during egg parasitism.

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Scale bar: 30 μ m.

Figure 5. Expression of *P. chlamydosporia* chitosanase encoding genes.

Relative expression of chitosanase encoding genes (GH75) of *P. chlamydosporia* (light blue) and *P. chlamydosporia* in presence of RKN eggs (dark blue) by qPCR. *csn1* (A), *csn3* (B), *csn4* (C), *csn5* (D), *csn6* (E), *csn7* (F), *csn8* (G), *csn9* (H) and *csn10* gene (I).

Significant effect of RKN eggs presence at $*P < 0.05$ and $**P < 0.01$ is indicated. Bars indicate mean \pm SD. N = 6.

Figure 6. Expression of *P. chlamydosporia* chitin deacetylase encoding genes.

Relative expression of chitin deacetylase encoding genes of *P. chlamydosporia* (light green) and *P. chlamydosporia* in presence of RKN eggs (dark green) by qPCR. *cda1* (A) and *cda2* (B) expression was ca. thirteen-fold and ca. three-fold, respectively, in presence of RKN eggs. Significance at $*P < 0.05$ and $**P < 0.01$ is indicated. Bars indicate mean \pm SD. N = 6.

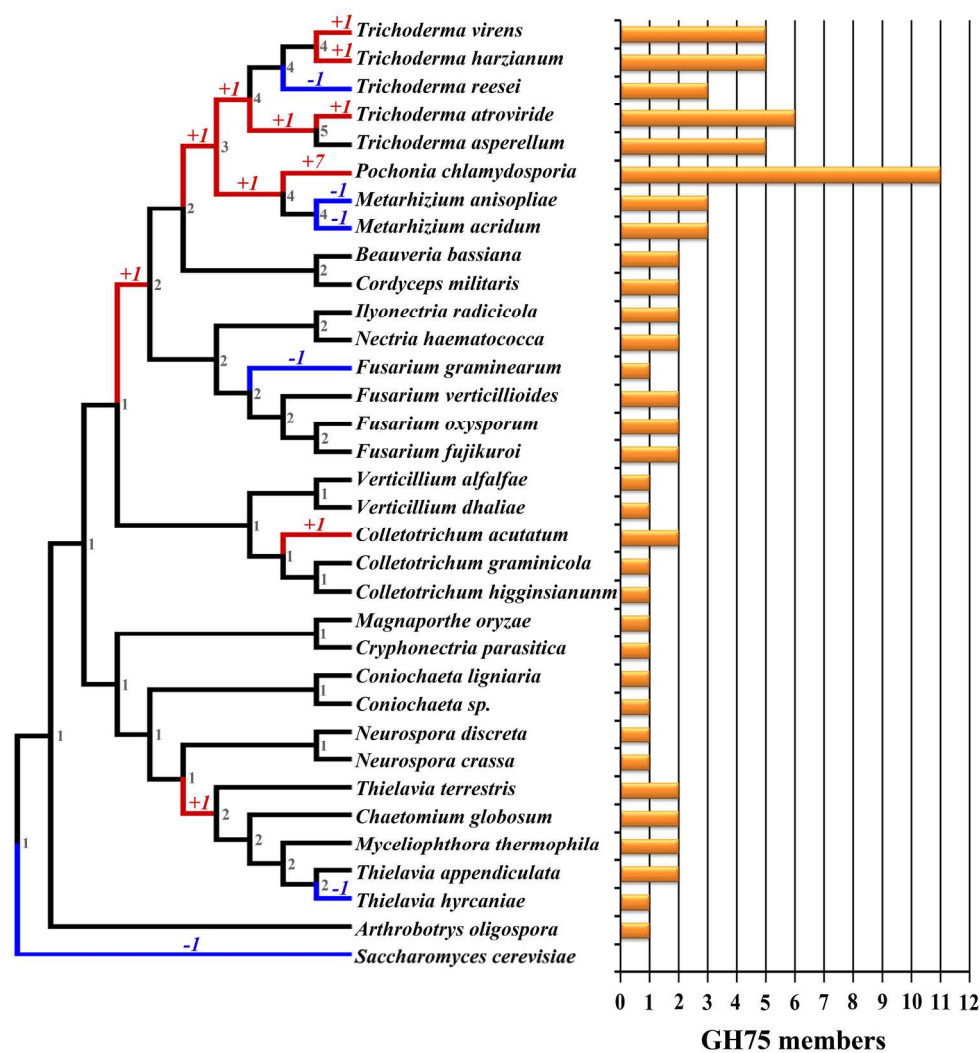


Figure 1. Evolutionary gain and losses in family GH75 chitosanases.

210x223mm (300 x 300 DPI)

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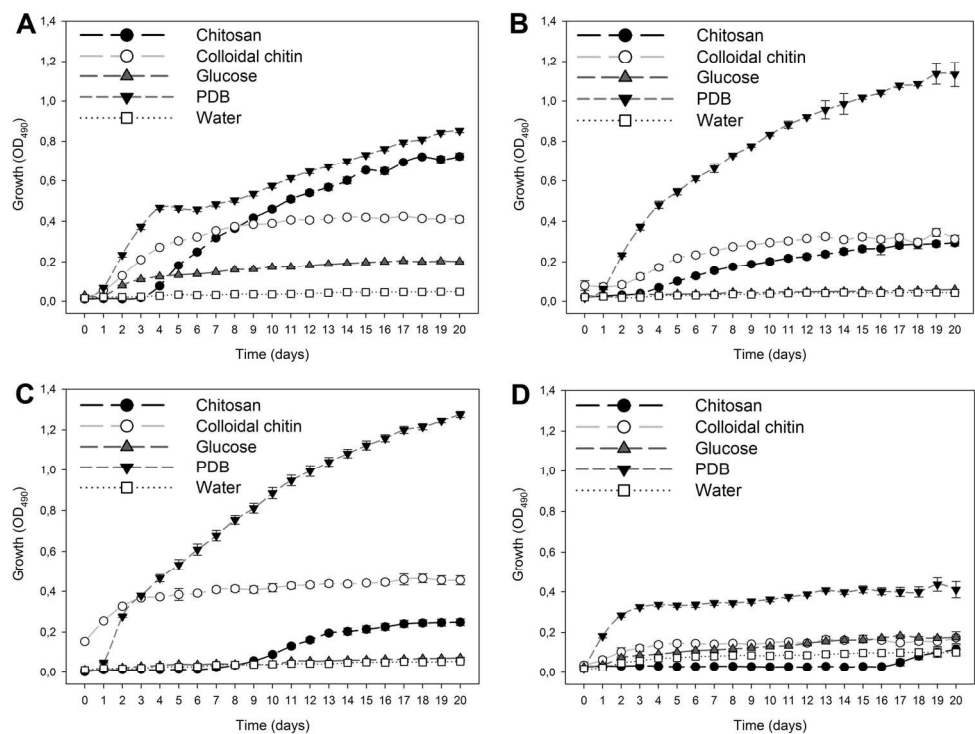


Figure 3. Colloidal chitin and chitosan as nutritional substrates.

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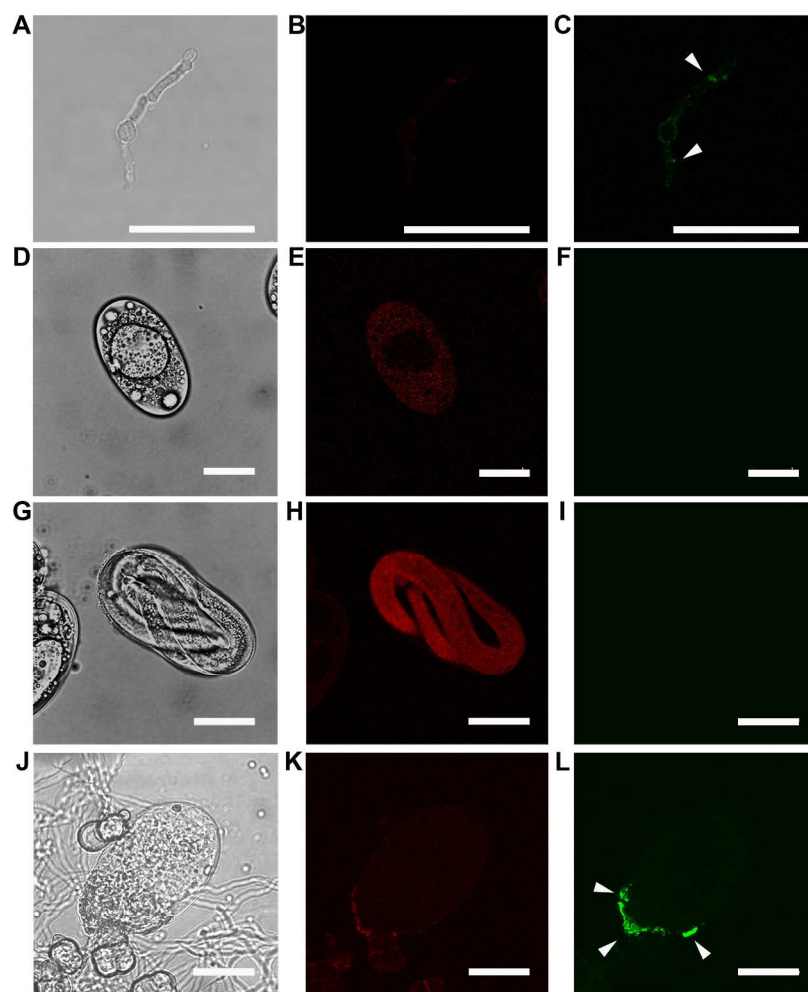


Figure 4. Chitosan immunolocalization.

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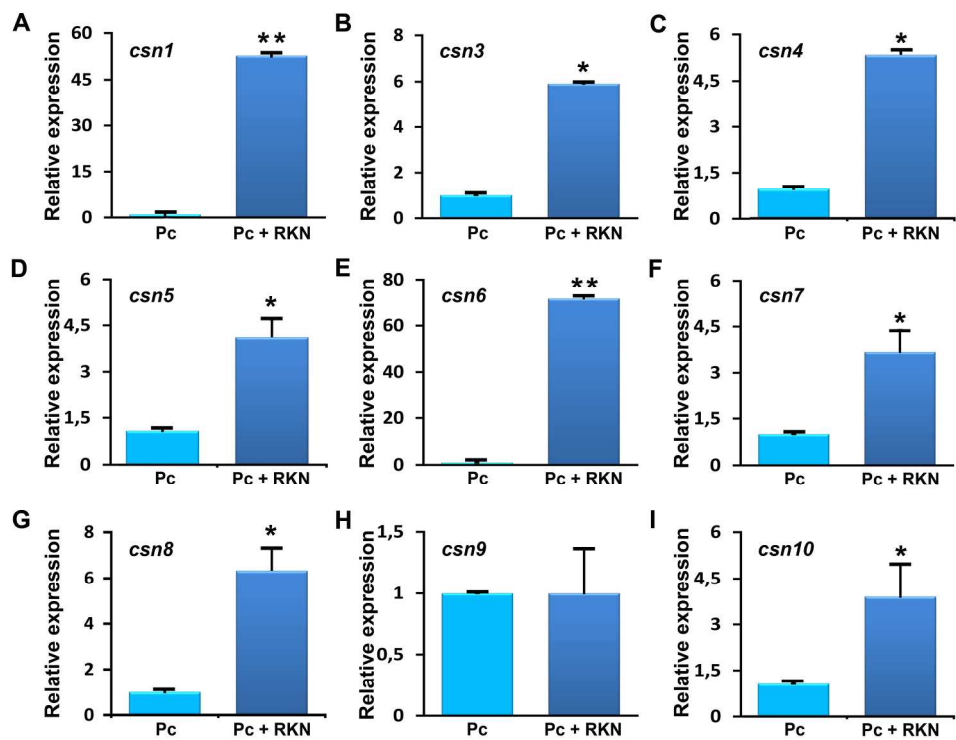


Figure 5. Expression of *P. chlamydosporia* chitosanase encoding genes.

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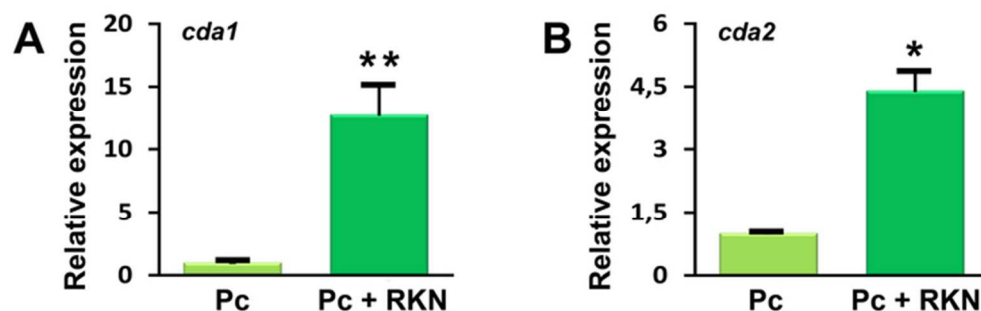


Figure 6. Expression of *P. chlamydosporia* chitin deacetylase encoding genes.

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